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Affinity of lipophilic drugs to mixed lipid aggregates in simulated gastrointestinal fluids

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Abstract

Mixed lipid aggregates, comprising of bile salts and phospholipids, present in the small intestine assist in drug solubilization and subsequent drug dissolution and absorption through the intestinal epithelium. The increased variability in their levels, observed physiologically, may create challenges not only for *in vivo* bioavailability and bioequivalence studies, but also for *in vitro* bio-predictive studies as correlations between *in vitro* and *in vivo* data are not always successful. The current study investigated the impact of biorelevant dissolution media, with physiologically relevant sodium taurocholate and lecithin levels, on the apparent solubility and affinity of lipophilic compounds with a wide range of physicochemical properties (drug ionization, drug lipophilicity, molecular weight) to mixed lipid aggregates. Apparent solubility data in biorelevant dissolution media for the studied neutral drugs, weak bases and weak acids were compared against a phosphate buffer pH 6.5 in the absence of these lipidic components. Presence of mixed lipid aggregates enhanced the apparent solubility of the majority of compounds and the use of multivariate data analysis identified the significant parameters affecting drug affinity to mixed lipid aggregates based on the chemical class of the drug. For neutral drugs, increasing bile salt concentrations and/or drug lipophilicity resulted in greater enhancement in apparent solubility at 24-hr. For weak bases and weak acids, the effect of increasing bile salt levels on apparent solubility depended mostly on an interplay between drug lipophilicity and drug ionization.

Keywords: drug lipophilicity, ionization, solubilization, affinity, mixed lipid aggregates, biorelevant dissolution media

1. Introduction

The lipophilic nature of drug target proteins results in the identification of lipophilic compounds as successful drug candidates by high-throughput screening [1]. Although pharmacologically active, lipophilic drugs suffer from poor aqueous solubility which presents several absorption challenges for oral administration. Only dissolved drug molecules are capable of permeating through the gastrointestinal epithelium Low aqueous solubility can therefore impede oral drug absorption and subsequently oral drug bioavailability. Varying approaches are employed during product development to increase drug solubility and improve product performance (e.g. salt formation, crystal structure modification, use of surfactants, enabling formulations) [2]. *In vivo*, a number of gastrointestinal factors may influence drug solubility and improve (or impede) oral drug absorption. The pH of gastrointestinal fluids is critical for the solubility of ionizable compounds, as ionized molecules exhibit higher solubility than their neutral form. Presence of natural solubilizing components (e.g. bile salts, phospholipids, and ingested lipids) in gastrointestinal fluids play a key role in solubilizing lipophilic compounds and enhance their dissolution, solubility and finally absorption and bioavailability.

Bile salts are amphiphilic molecules present in the gastrointestinal tract with a key role in the digestion of dietary fat and absorption of lipophilic compounds [3]. Primary bile acids are produced by the hepatocytes, stored and converted to salts in the gall bladder, until they are secreted to the duodenum once acid chyme enters the small intestine [3]. A number of bile acids are produced *in vivo*, with cholic and chenodeoxycholic acid (further conjugated with glycine and taurine forming the corresponding bile salts) being the most predominant forms [4]. Bile salts are steroidal molecules and differ in the number, position and stereochemistry of the hydroxy groups in the steroid structure[3]. At low concentrations and due to their amphiphilic nature, bile salts act as surfactants facilitating the wetting of the administered formulation [5]. At

increased concentrations [above the critical micellar concentration (CMC)], bile salts self-assemble into aggregates (spherical, rod or lamellar shaped) [3] and can solubilize lipophilic compounds. A suggested mechanism by which bile salt aggregates are formed includes a stepwise process in which the hydrophobic parts come in contact exposing the hydrophilic head to the solvent (formation of primary micelles) [6]. Primary micelles can further aggregate into secondary aggregates via intermicellar H-bonding among the hydrophilic parts [6]. In the presence of natural phospholipids (e.g. phosphatidylcholine, lysolecithin, phosphatidylglycerol, diphosphatidylglycerol, sphingomyelin) [7], which are secreted with the bile into the duodenum, mixed lipid (bile salt:phospholipid) aggregates are formed which exhibit greater stability and solubilizing capacity compared to aggregates comprising only bile salts.

The composition, levels and structure of mixed aggregates *in vivo* show high variability, due to inter/intra subject variations or certain disease states. This variability affects the solubilizing capacity of these aggregates may influence oral drug bioavailability. Structurally differing bile salts are excreted into the small intestine. Studies evaluating the composition of human intestinal fluids report taurocholic, taurochenodeoxycholic, taurodeoxycholic, glycocholic, glycochenodeoxycholic and glycodeoxycholic salts as the main bile components present in the duodenum [8]. It has been suggested that the varying degree of hydroxylation of these bile components may influence the solubilizing capacity of the formed mixed lipid aggregates [7,9]. Regional differences in the bile salt levels are known. Bile salts are primarily present in the small intestine, while small amounts (1 mM) in stomach (in the fasted or fed state) due to duodenal reflux have been reported in literature [10]. In the fasted duodenum, the bile salt concentrations vary but are significantly higher than the ones in the stomach. Large variations in their level are found in human aspirates ranging between 0.03-36.18 mM [8] with mean and median values in the average range of 1.4-8.1 mM [8]. Post meal administration, the

bile salt concentrations rise even further and range between 0.74-86.14 mM [8] with mean and median values in the range of 3.6-24 mM [8]. As the solubilizing capacity of mixed lipid aggregates (and also drug solubility) increases as a function of taurocholate and lecithin concentration, variations in their levels are rendered critical *in vivo* and need to be accounted for in *in vitro* experimental setups. For patients with gastrointestinal diseases, changes in the bile salts concentrations are also known [11]. The supramolecular structure of the lipidic phase itself may vary *in vivo*. Characterization of human aspirates revealed the presence of micelles of diverse composition and distinct size fractions [pure cholate or cholate-rich micelles (< 20 nm) and mixed cholate/phospholipid micelles or phospholipid vesicles (50-200 nm)] [12,13]. The solubilizing capacity of these different phases varies and may significantly oral drug absorption [14].

As the use of human aspirates for *in vitro* experimentation to assess product performance is not always feasible or practical, due to availability, cost and variability, biorelevant media have been developed to reflect the composition of different gastrointestinal fluids. These media are constantly reviewed and updated to better reflect *in vivo* conditions in terms of pH, buffer capacity, osmolarity, composition and concentration of amphiphilic components (sodium taurocholate, lecithin) [15,16]. Although each update improves the biorelevance of these media, deviations in drug solubility values between simulated fluids and human aspirates still prevail [17]. The observed increased variability in the concentrations of certain gastrointestinal components *in vivo* has been a matter of concern lately [18-20] and may explain the *in vitro-in vivo* discrepancies in drug solubility.

The aim of this study was to investigate the affinity of lipophilic compounds to mixed lipid aggregates present in gastrointestinal fluids. The impact of mixed lipid aggregates on drug apparent solubility was studied in a biopharmaceutical perspective. Biorelevant dissolution media, with varying levels of amphiphilic components, were used to assess drug's affinity to

the micellar phases simulating the fasted state gastrointestinal conditions. Drug-specific interactions with mixed lipid aggregates were investigated by selecting lipophilic compounds with varying physicochemical (drug ionization, $logD_{pH6.5}$, molecular properties) and molecular properties. Multivariate data analysis [Partial Least squares (PLS)] was used to identify the critical drug properties affecting compound solubilization and partitioning to the lipidic mixed aggregates.

2. Materials and Methods

2.1 Materials

APIs: Carvedilol, corticosterone, dipyridamole, indoprofen, naproxen, and warfarin were obtained from Sigma-Aldrich, UK. Astemizole, cinnarizine, griseofulvin, haloperidol, indomethacin, tamoxifen and tolfenamic acid were purchased from Alfa Aesar, UK. Disopyramide was purchased from MP biomedicals, UK. Progesterone was purchased from Merck Millipore, UK **Chemicals:** High Performance Liquid Chromatography (HPLC) methanol and acetonitrile were purchased from VWR, UK. Sodium chloride and sodium hydroxide were acquired from Fisher Scientific, UK. The Fasted State Simulated Intestinal Fluid (FaSSIF)/Fed State Simulated Intestinal Fluid (FeSSIF)/Fasted State Simulated Gastric Fluid (FaSSGF) powder was purchased from Biorelevant, UK. Water was ultra-pure (Milli-Q) laboratory Grade. **Consumables:** Syringe filters Polytetrafluoroethylene (PTFE) 0.45 µm pore size (Fisherbrand, UK) and Cronus Regenerated Cellulose (RC) 0.45 µm pore size (LabHut, UK) were obtained from the specified sources. **Instrumentation:** Fisherbrand waterbath (Fisher Scientific, UK), Mettler Toledo SevenCompact S210 pH meter (Schwerzenbach, Switzerland), Agilent Technologies 1200 series HPLC system (Santa Clara, CA): binary pump (G1311A), autosampler (G1329A), thermostatted column compartment (G1316A), and diode array detector (G1315A).

2.2. Methods

2.2.1 Compound selection for solubility studies

The selected compounds for the apparent solubility studies are presented in **Table 1**. Compounds were selected to cover a wide range of physicochemical properties associated with drug affinity to mixed lipid aggregates [21]. Drug were categorized based on their drug ionization on pH 6.5 (low ionization: F_{ion} <50%, high ionization: $F_{ion} \geq 50\%$), drug lipophilicity [low lipophilicity: log $D_{pH\ 6.5}$ (logarithm of the octanol:water partition coefficient at pH 6.5) < 0, medium lipophilicity: $0 \leq \log D_{pH\ 6.5} < 2$, high lipophilicity: $\log D_{pH\ 6.5} \geq 2$] and molecular weight (medium molecular weight: $200 \leq MW < 500$, high molecular weight: $MW \geq 500$). The structure of the studied compounds is presented in **Figure 1**. The molecular descriptors (polar surface area, rotatable bonds, hydrogen bond donors and hydrogen bond acceptors) of the studied compounds are presented in **Supplementary Table 1**.

2.2.2. Preparation of solubility media

The composition of the solubility media is presented in **Table 2**. The phosphate buffer ($PhB_{pH\ 6.5}$), not containing bile salts, was prepared at room temperature by dissolving 0.420 g of NaOH, 3.438 g of NaH_2PO4 and 6.186 g of NaCl in 0.9 L of deionized water. The pH was adjusted to 6.5 and the volume was filled up to 1 L with deionized water. The biorelevant dissolution media (BDM), containing different bile salt and phospholipid concentrations, were prepared by dissolving SIF powder to the $PhB_{pH\ 6.5}$. All SIF media were freshly prepared and allowed to stand for 2 hr, to achieve equilibration [22], in room temperature until a slightly opalescent micellar solution was obtained. The studied bile salt levels were selected to reflect the physiological variability e.g. i. Biorelevant dissolution media with low taurocholate and lecithin concentrations (BDM_L): achlorhydric conditions in the fasted stomach and ii. Biorelevant dissolution media with medium (BDM_M) and high (BDM_H) mixed lipid aggregate

concentrations: media covering closely the low and upper limits in the mean and median bile salt levels reported at fasted state *in vivo*, as explained in Section 1. Studies suggest that the CMC of taurocholate lies between 1-12 mM or that taurocholate exhibits no strict CMC and forms smaller aggregates at increasing levels [22,23]. Under the studied concentrations therefore, mixed lipid aggregates, rather than micelles, are expected to be formed in the BDMs.

2.2.3. Solubility studies

Drug solubility studies in the $PhB_{pH\ 6.5}$ and BDMs were performed in triplicate using the shake-flask method [24]. An excess amount of drug (approximately three times higher than the estimated equilibrium drug solubility) was weighed in labelled eppendorf tubes. 5 mL of each solubility medium was added in the tubes. The samples were vortexed and placed in a shaking water bath [37 °C, 200 strokes per minute (spm)]. To be noted, that stability issues of the mixed lipid aggregates are not expected when heating the BDMs at 37°C for 24 hr [22]. At 24 hr, 500 μL were sampled and filtered through PTFE filters. Filter adsorption studies were prior performed in triplicate for each drug. No adsorption issues onto the filters used were observed for the studied drugs. Filtered samples were further diluted (if needed) with the corresponding mobile phase and analysed by HPLC-UV. Analytical HPLC procedures were based on modification of methods taken from the literature **(Supplementary Table 2**). Drug quantification was made based on calibration curves. Standards were prepared from concentrated stock solutions consisting of drug dissolved in an organic solvent.

2.2.4 Data Treatment

Solubility data at 24-hr in $PhB_{pH6.5}$ were expressed in molarity. Differences in the apparent drug solubility between the $PhB_{pH\ 6.5}$ and the BDMs were expressed as the logarithm of the Solubility Ratio (logSR), calculated based on Equation 1

$$logSR = log\left(\frac{S_{BDM}}{S_{PhB6.5}}\right) \qquad \text{Equation 1}$$

where S_{BDM} and $S_{PhB6.5}$ denote drug apparent solubility in the BDM and $PhB_{pH\ 6.5}$, respectively at 24 hr. logSR< 0, logSR= 0 and logSR> 0 indicate reduced, equal and higher, respectively, apparent drug solubility in the BDM when compared to $PhB_{pH6.5}$.

Graphs depicting the logSR of the mixed aggregates on drug apparent solubility at 24-hr were constructed using GraphPad Prism (GraphPadPrism, USA). Linear regressions of drug apparent solubility (μM) as a function of bile salt concentrations (mM) were performed using GraphPad Prism (GraphPad Prism, USA), and the slopes and R^2 of each regression line were calculated automatically by the software.

Hansen solubility parameters, describing solutes and solvents in terms of intermolecular dispersion forces (δD), polarity forces (δP) and hydrogen bond forces (δH), were calculated for all studied compounds, taurocholate and lecithin from their SMILES structures using the Yamamoto molecular break (Y-MB) tool in HSPiP [25]. The Y-MB tool uses a group contribution routine to calculate Hansen parameters based on molecular structure. Hansen parameters for a lipid aggregate phase were calculated based on the molar ratios of taurocholate and lecithin (4:1) in the BDM assuming that all lipids are present in the lipid phase and omitting the possibility that a fraction of the taurocholate is involved in smaller aggregates or as monomers in the aqueous phase. The Hansen distance (ΔHansen), as a measure on similarity between solvent and solute, was calculated based on the solubility parameters for drug-water ($\Delta Hansen_{water}$) and drug-mixed lipid aggregate ($\Delta Hansen_{MLA}$) according to Equation 2 [25].

$$\Delta Hansen = \sqrt{\begin{matrix} 4 \times (\delta D_{Solute} - \delta D_{Solvent})^2 \\ + \ (\delta P_{Solute} - \delta P_{Solvent})^2 \\ + (\delta H_{Solute} - \delta H_{Solvent})^2 \end{matrix}} \qquad \text{Equation 2.}$$

A low ΔHansen would indicate similarity between the drug and the media and therefore result in a higher solubility, while a high ΔHansen indicates that the solvent and solute are not

similar leading to a lower solubility, or solubilization in the case of drug solubilization in mixed lipid aggregates present in BDM.

2.2.5 Statistical Analysis

The logSRs for the drugs in each BDM were correlated to drug physicochemical properties (drug ionization, drug lipophilicity, molecular weight) and media characteristics (bile salt levels) by partial least squares (PLS) regression using the XLSTAT software (Microsoft, USA). Three models for the logSR of the neutral drugs (Model 1), weak bases (Model 2) and weak acids (Model 3) in the BDM were constructed. The evaluated variables were all numerical: i. bile salt concentration (BS), ii. % of drug ionized (F_{ion}; calculated based on the Henderson – Hasselbalch equation at the pH of 6.5), iii. drug lipophilicity (log $D_{pH\ 6.5}$), iv. molecular weight (MW) and v. melting temperature (T_m, **Table 1**). The calculated logSR (section 2.2.4) was set as response. The selected interaction terms included the level of bile salts combined with each drug physicochemical property (drug ionization, drug lipophilicity, molecular weight, melting temperature). The generated models were assessed in terms of goodness of prediction (Q^2) and goodness of fit (R^2). $Q^2 > 0.5$ [26] and R^2 and Q^2 values with a difference not greater than 0.2 - 0.3 [27] were indications of acceptable models. The number of PLS components (lines on the X-space which best approximate and correlate with the Y-vector) was selected as described previously [24]. Standardized coefficients were used to show the direction (positive or negative) and extent of each variable on the response. The significance of the variables was assessed by the variable influence on projection (VIP) value. VIP values > 0.8 were considered as moderately influential in the model while VIP values > 1 were considered the most influential in the model [27]. A 95 % confidence interval was used.

To further explore molecular properties related to drug-mixed lipid aggregate affinity and solubilization, more than 80 descriptors were calculated using HSPiP, DataWarrior [28], Chemaxon Excel [29] and SwissADME [30]. Descriptors without information and duplicate

descriptors (i.e MW from 3 out of 4 software) were removed. The 64 remaining calculated descriptors were, together with $clogp_{pH6.5}$, assessed for correlation to logSR for all compounds in BDM_H.

3.Results and Discussion

3.1 Solubility studies

The apparent solubility values of the studied drugs in $PhB_{pH\ 6.5}$ are presented in **Table 1** (for all compounds, apparent solubility values in $PhB_{pH\ 6.5}$ have been experimentally determined for the purposes of this study, except from the values of danazol, astemizole and carvedilol, which have been previously determined experimentally by the authors [31]).

Neutral drugs: A range of apparent solubility values was observed (1.8-324 μM) in $PhB_{pH\ 6.5}$ explained by the varying lipophilicity of the studied neutral compounds; highly lipophilic drugs exhibited lower apparent solubility at 24 hr [32]. Only progesterone slightly deviates from the above observation, despite its high lipophilicity which can be attributed to its lower molecular weight [32] or low melting temperature (**Table 1**). The effects of media with various bile salt levels on drug apparent solubility are shown in **Figure 2**. For this set of compounds, an enhancement in drug apparent solubility ($0.18 < logSR < 1.77$) was observed in the presence of lipidic components, due to their solubilization capacity [5]. To be noted that for corticosterone in BDM_L and BDM_M (logSR of approximately -0.10 in both media) and for danazol in BDM_L (logSR = -0.17), the data reveal that the apparent solubility values in the aforementioned cases were lower when compared to the $PhB_{pH\ 6.5.}$ This can be attributed to interday (corticosterone) or interlaboratory (danazol) variability. The fact that apparent solubility values in BDM_L for both compounds and in BDM_M for corticosterone were not anticipated to greatly differ as compared in $PhB_{pH\ 6.5}$ due to the low bile salt concentrations and the lipophilicity of corticosterone which lies in the lower limit (≈2) of the high lipophilicity criterion (**Table 1**),

255 respectively, is considered a contributing factor for the observed logSR. When comparing the
256 results within the different BDMs, a general trend was observed with greater solubility
257 enhancement for danazol, progesterone and felodipine than corticosterone and griseofulvin
258 (**Figure 2**). As the studied neutral compounds are unionized and comprise of a narrow range
259 of molecular weight (MW ≈ 350), it is reasonable to conclude that the above finding relates to
260 the differences in drug lipophilicity; danazol, progesterone and felodipine (log $D_{pH\ 6.5} > 3$)
261 benefit more from the presence and increased concentrations of mixed aggregates. Assuming
262 that the theory of drug solubilization in mixed aggregates is governed by the two-state model
263 (in which aggregates are considered a separate phase and drug partitioning resembles the one
264 between a bulk organic solvent and water [33]), the impact of drug lipophilicity in the
265 solubilization process is not surprising.

266 **Weak bases:** As per neutral compounds, the apparent solubility values of weak bases in PhB_{pH}
267 $_{6.5}$ varied (1.1-2312.8 μM) as the studied compounds comprised of a wide range of
268 physicochemical properties (**Table 1**). The differences in the apparent solubility values are
269 mostly explained by the differences in drug lipophilicity, as highly lipophilic compounds
270 exhibited, in general, lower apparent solubility [32]. Only for dipyridamole and astemizole,
271 apparent solubility values at 24 hr were considerably low which is likely explained by their
272 pKa and low ionization in the studied medium. Presence of bile salts in BDM_L ($0.07 < logSR$
273 < 0.58), BDM_M ($0.20 < logSR < 1.98$) and BDM_H ($0.36 < logSR < 2.77$) enhanced the apparent
274 solubility of all studied weak bases. For dipyridamole (logSR = -0.01) and astemizole (logSR
275 = -0.18), a reduction in the apparent solubility was observed in BDM_L attributed to similar
276 factors (e.g. variability) as explained for neutral drugs. Understanding the effects of bile salt
277 presence on the apparent solubility of weak bases requires a more profound look on the
278 physicochemical drug properties. For the highly ionized drugs, the impact of bile salts on drug
279 apparent solubility seems to differ for drugs with log $D_{pH\ 6.5} < 0$ (disopyramide), log $D_{pH\ 6.5} \approx$

2 (carvedilol, haloperidol) and log $D_{pH\ 6.5}$ > 4 (cinnarizine, tamoxifen). For disopyramide, although apparent solubility values were higher in the BDMs compared to $PhB_{pH\ 6.5}$, pronounced differences in drug apparent solubility when increasing the bile salt levels were not observed (**Figure 2**). For carvedilol and haloperidol increasing the bile salts resulted in an improvement in drug apparent solubility [logSR up to approximately 0.56] but not to the same extent as for the cinnarizine and tamoxifen for which logSR were profoundly high, especially in BDM_M and BDM_H [0.81 < logSR <2.77). Mixed aggregates can bind ionized and unionized drugs but at different binding constants [34]. Ionized weak bases should be able to bind to the negatively charged mixed aggregates [35] at some extent due to their positive charges. An interplay is observed between drug ionization and drug lipophilicity, in this study, as highly lipophilic and highly ionized weak bases will benefit more with increasing bile salt levels when compared to low lipophilic but highly ionized weak bases. The high driving force for partition in lipidic phases of highly lipophilic compounds seems to diminish the unfavourable effects of drug ionization for partition in mixed aggregates. For drugs that are poorly ionized in the studied media (dipyridamole, astemizole), the increase in drug apparent solubility in BDM_M and BDM_H is similar or even greater (**Figure 2**) when compared to highly ionized drugs of low or average lipophilicity. Although for astemizole, the observation is not surprising due to the high drug lipophilicity ($logD_{pH6.5}$ = 4.4), it is of particular note for dipyridamole which is a compound of average lipophilicity (log $D_{pH6.5}$ = 1.8) and should not exhibit higher logSR when compared to the other two weak bases of average lipophilicity (carvedilol, haloperidol). The dipyridamole example demonstrates, therefore, the importance of drug ionization on the affinity of drugs to mixed lipids; drugs of average lipophilicity will still partition to a great extent into mixed aggregates when they are not completely ionized.

Weak acids: Similarly to the other two chemical drug classes, a wide range of apparent solubility values was found for weak acids (126-3438 μM) and as a general trend compounds

with log $D_{pH\ 6.5} > 2$ exhibited lower apparent solubility as compared to compounds with log $D_{pH\ 6.5} < 2$ (**Table 1**). Improvement in drug apparent solubility was observed in the BDMs for all studied compounds [$0.04 < logSR < 0.60$) except from the cases of indomethacin in BDM_M (logSR== -0.04±0.02) and of warfarin in BDM_L [logSR = -0.54±0.04] and BDM_M [logSR = -0.20±0.01) (**Figure 2**). The pronounced reduction in warfarin apparent solubility, especially in SIF_L, raises questions on whether this observation relates solely to experimental variability and requires further investigation; nonetheless warfarin was not excluded from this data set as it represents the most lipophilic weak acid and a clear dependency of drug apparent solubility at 24 hr on bile salt concentration was observed. As per weak bases, two distinct cases were found in which experimental findings can be discussed: i. weak acids with log $D_{pH\ 6.5} < 2$ (indomethacin, naproxen, indomethacin) and ii. weak acids with log $D_{pH\ 6.5} > 2$ (tolfenamic acid, warfarin). In the former case, the improvement in drug apparent solubility in presence of bile salts was minor [logSR up to approximately 0.11] and increase in bile salts levels did not greatly improve apparent drug solubility. Weak acids are negatively charged in the studied media and their negative charge is the predominant factor in drug partition to mixed aggregates when compared to their lipophilicity [36]. In the latter case, drug apparent solubility was greatly improved [$0.21 < logSR < 0.60$] in BDM_M and BDM_H (except from the case of warfarin in BDM_M). This observation once more illustrates a complex interplay between drug ionization and drug lipophilicity in the affinity of drug to mixed aggregates, as explained for the weak bases.

The solubility studies revealed that drug concentrations can greatly be increased in media simulating the gastrointestinal fluids when solubilizing components are of concern. The extent of apparent solubility improvement depends on the physicochemical properties of active compounds; a dependency that needs to be properly delineated and accounted for. A number of physicochemical properties are considered critical for the affinity of drug to mixed lipid

aggregates (e.g. molecular size, charge, polarity), with lipophilicity being the most influential one [21]. Predictive models to understand the solubilization capacity of bile salts based on drug lipophilicity have been developed [5]. However it has been shown that drug lipophilicity solely is not always sufficient to explain the observed solubilization effects [37]. The findings of this study demonstrate that the critical physicochemical properties dictating drug partitioning into mixed aggregates may differ according to drug chemical class. For neutral compounds lipophilicity explained majority of the effects of mixed lipid aggregates on drug apparent solubility, but for weak bases and weak acids, a strong interplay between drug lipophilicity and drug ionization was portrayed. Interestingly, it is noted that despite the beneficial role of bile salts in drug solubilization, attention is drawn as the profound differences in drug apparent solubility indicate that variations in the levels of these lipidic components *in vivo* can induce immense changes in the concentrations of drugs in the gastrointestinal lumen that could harm the patient (e.g. toxic drug levels in bloodstream) or impose regulatory challenges in product approvals (e.g. bioequivalence studies).

3.2. Affinity of lipophilic compounds to mixed lipid aggregates in simulated intestinal fluids

Hansen solubility parameters were calculated for all studied drugs, water, pure taurocholate, pure lecithin and finally mixed lipid aggregates. To assess the drugs affinity, or preference, between the aqueous phase and mixed lipid aggregate phase, ΔHansen were calculated based on the difference in the parameters between solutes and solvents (water and mixed lipid aggregates. Parameters and ΔHansen are presented in **Table 3**. To summarize the results, all studied drugs had higher $\Delta Hansen_{water}$ than $\Delta Hansen_{MLA}$ indicating that they are more similar to the mixed lipid aggregates than the aqueous phase. This is not surprising given their relatively high MW and lipophilic nature. Dipyridamole was the most water like drug

with the datasets lowest $\Delta Hansen_{water}$ of 33.6. Corticosterone, that indeed has a similar molecular structure as taurocholate, was the compound with the lowest $\Delta Hansen_{MLA.}$ Cinnarizine and progesterone were the compounds with the highest $\Delta Hansen_{water}$ indicating that they are least water like compounds. Cinnarizine was also the drug with highest $\Delta Hansen_{MLA,}$ or least similar to the mixed lipid aggregates. An interesting result was the large difference in $\Delta Hansen_{water}$ for progesterone (41.4) and corticosterone (33.6) with similar molecular structures (**Figure 1**). The two additional hydroxyl groups in the latter affect both the p and h parameter strongly.

In an attempt to better outline the affinity of lipophilic compounds in mixed lipid aggregates in the studied BDMs, apparent solubility data at 24 hr have been plotted and regressed against the levels of bile salts. The regression plots for neutral drugs, weak bases and weak acids are presented in **Figures 3, 4 and 5**, respectively. The regression plots do not take into account the solubility data in $PhB_{pH\ 6.5}$; regression lines with the solubility data in $PhB_{pH\ 6.5}$ have been generated and reduction in the goodness of fit (reduction in the R^2 ranged between approximately 0.0001-0.3000 units) was observed for the majority of models. This was expected as large differences between the apparent solubility values in $PhB_{pH\ 6.5}$ and BDM_L were not expected due to the low bile salt levels in the latter medium; data interpretation and conclusions drawn from these plots do not change.

For neutral drugs, a linear dependency between the increase in the apparent drug solubility and bile salt concentrations was observed with high values of goodness of fit ($R^2 > 0.89$) (**Figure 3**). The ability of bile salts to form mixed aggregates with lecithin depends on the concentration of these lipidic components and it is known that at the levels introduced in BDM_L (medium simulating the bile salt/lecithin concentrations in the achlorhydric stomach) predominantly the wetting abilities of bile salts prevail [5]. Mixed lipid aggregates (as explained in section 2.2.2.),

able to solubilize lipophilic compounds, are expected to be formed in the other two simulated media and explain the pronounced observed increase in drug solubility. The extent of solubilization in mixed aggregates strongly depends on the properties of both bile salts (e.g. concentration) and drug (e.g. size, charge and lipophilicity) [21]. As the studied neutral compounds have an average molecular weight, are uncharged and exhibit average or high lipophilicity, their solubilization should mainly depend on the bile salt levels. As already reported in literature, the presence of more solubilizing components in the medium increases the apparent solubility of neutral compounds [34]. The observed linear dependency indicates that, at least at the studied range of bile salt levels (0.08-7.5 mM), there are no confounding factors affecting the solubilization of neutral drugs in mixed aggregates; the bile salt levels, along with drug lipophilicity, mainly dictate drug solubilization.

For weak bases, a linear dependency between drug apparent solubility and bile salt concentrations is observed for the majority of compounds (R^2 > 0.85 for dipyridamole, astemizole, carvedilol, haloperidol, tamoxifen) (**Figure 4**). For these compounds, it is evident that their diffusion and partition into the mixed aggregates depends on the concentration of the lipidic components, as per the neutral compounds. Although, the aforementioned drugs are partially (dipyridamole, astemizole) or fully ionized (carvedilol, haloperidol, tamoxifen) in the studied media, it is noted that due to the negative net charge of aggregates, weak bases will be adequately solubilized in the aggregates, despite their ionization [34]. In two cases, regression fits were poor (disopyramide, R^2 = 0.25) or moderate (cinnarizine, R^2 = 0.65). For disopyramide, the data can be attributed to the hydrophilic nature of the ionized species (log $D_{pH\,6.5}$ = -0.3) suggesting that variation in bile salts is not a critical parameter for highly ionized poorly lipophilic drugs. Based on the generated dataset, a good correlation between the increase in apparent solubility values of cinnarizine and the increase in bile concentrations was anticipated although not observed due to its high lipophilicity ($logD_{pH6.5}$ = 4.3). Additional

investigations are required to explain the behavior of this drug, as none of its physicochemical properties (molecular weight, ionization, lipophilicity) can explain the lack of a good fit. Cinnarizine is the studied compound with the highest calculated $\Delta Hansen_{MLA.}$ The large difference between cinnarizine's and the mixed lipid aggregate solubility parameters indicate a poor solute-solvent match despite the drugs high lipophilicity which could relate to the comparatively low observed solubilization. Furthermore, the central and shielded location of cinnarizine's basic nitrogens (**Figure 1**) may reduce the possibility of electrostatic interaction with the negatively charged lipid aggregates and may also be a reason for the lack of good fit between lipid contents and solubilization.

For weak acids, poor correlations were observed for indoprofen (R^2 = 0.46), naproxen (R^2 = 0.64) and indomethacin (R^2 = 0.59) (**Figure 5**). The aforementioned compounds comprise of average lipophilicity ($0.7 < logD_{pH6.5} < 1.3$) and are completely ionized in the studied biorelevant media. This is not of surprise as these negatively charged molecules cannot easily diffuse through the negatively charged aggregates [34]. On the contrary, a good correlation between drug and bile salt concentration was only observed for tolfenamic acid (R^2 = 0.99) and warfarin (R^2 = 0.99). Despite their ionization and negative charge, these two compounds are very lipophilic ($log\ D_{pH\ 6.5} \geq 2$). It is evident therefore, that the impact of drug ionization on drug affinity to the mixed aggregates is diminished and the beneficial impact of drug lipophilicity on drug partitioning into the mixed aggregates prevails in this case. This investigation for weak bases and weak acids reveals that a more complex interplay between bile salt levels and drug physicochemical properties on drug solubilization exists.

3.3. Multivariate Data Analysis

PLS was conducted to delineate the key biopharmaceutical factors affecting the affinity and solubilization of drugs in mixed aggregates. These models are not used as predictive, due to the absence of validation methods, but mainly to understand how different drug classes are solubilized in the mixed lipid aggregates of the studied media. The standardized coefficients of the variables in each model (neutral compounds, weak bases, weak acids) are presented in **Figure 6**. For neutral drugs, the model showed high goodness of prediction (Q^2 = 0.90) and fit (R^2 = 0.95) which is an indication that the studied biopharmaceutical variables adequately explain the affinity of lipophilic compounds to mixed aggregates. Log $D_{pH=6.5}$ (positive effect, VIP = 2.06), MW (positive effect, VIP = 1.46), BS (positive effect, VIP = 1.16) and T_m (negative effect, VIP = 1.01) were the significant variables in this model. The significance of log $D_{pH=6.5}$ confirms that highly lipophilic drugs have higher affinity and are better solubilized by bile salts, due to the lipophilic nature of the endogenous components of the gastrointestinal tract. Surprisingly, the model indicates that neutral drugs of higher MW will, as well, be greatly solubilized in the lipid aggregates. Although this finding tackles fundamentals aspects, as larger compounds are not expected to be adequately solubilized [38], it should be noted that the studied neutral drugs comprised of average molecular weight (MW ≈ 300-400) and that this conclusion should not be extrapolated to larger molecules (MW > 400 - 500). Increasing the levels of mixed lipid aggregates in the medium will significantly increase the solubility of neutral compounds, as indicated by the significance of the variable BS. Finally, the negative effect of the T_m variable suggests that the solubility of compounds with high crystal lattice energies will be less influenced (increase) by the presence of bile salts. For weak bases, the model showed adequate goodness of prediction (Q^2 = 0.51) and fit (R^2 = 0.65) revealing that the studied biopharmaceutical variables included can partially explain the observed effects of bile salts on drug apparent solubility. Log $D_{pH=6.5}$ (positive effect, VIP = 1.46), BS (positive effect, VIP = 1.34), T_m (negative effect, VIP = 1.31), MW (negative effect, VIP = 1.13) and $F_{(ion)}$ (positive

effect, VIP = 1.03) were the most influential main variables in the model. The statistical model reveals that the enhancement in drug solubility will be more pronounced for highly lipophilic weak bases and with increasing levels of bile salts, as indicated by the significance of the log $D_{pH=6.5}$ and BS variables, respectively. In addition, it is suggested that molecules with strong intermolecular forces or of larger size will benefit in a lower extent by the solubilizing capacity of the bile salts. In the case of weak bases, highly ionized molecules can still be solubilized by mixed lipid aggregates (due to the negative net charge of the latter, as explained in section 3.1) and this is considered a significant factor by the model. Understanding the affinity of weak bases to mixed aggregates is more complex, when compared to neutral drugs, as indicated by the significance of the interaction terms BS*log$D_{pH\ 6.5}$ (positive effect, VIP = 0.92). This interaction term reveals an interplay, as the enhancement in drug solubility by increasing the bile salt levels will be more pronounced for high, as compared to low, lipophilic compounds. Finally, for weak acids the model showed adequate goodness of prediction (Q^2 = 0.82) and fit (R^2 = 0.89). BS (positive effect, VIP = 1.57) and log$D_{pH\ 6.5}$ (negative effect, VIP = 0.82) were the most critical main variables in the model, while BS*$F_{(ion)}$ (negative effect, VIP = 1.39), BS*log $D_{pH\ 6.5}$ (positive effect, VIP = 1.19) and BS*T_m (positive effect, VIP = 0.86) were the most influential interaction terms. The conclusions drawn by the significance of the BS match the ones observed for weak bases. The negative effect of the log$D_{pH\ 6.5}$ variable needs to be interpreted with caution, as it may reflect the case of warfarin for which significant low log(SR) were observed. The model was able to capture the interplay of bile salt levels and drug lipophilicity (as revealed by the BS*log $D_{pH\ 6.5}$ interaction term) as increasing the bile salt levels will enhance the solubilization of highly lipophilic compounds to mixed aggregates. For weak acids, increasing their ionization will diminish the positive effects (increase) of bile salts on drug solubility, as due to their negative charge, weak acids are less likely to be solubilized by the mixed lipid aggregates. Interestingly, it was revealed that high levels of bile salts will

significantly enhance the solubility of compounds with high T_m, as indicated by the significance of the BS*T_m interaction term. Among the calculated descriptors associated with high SR in BDM_H, most were linked to lipophilicity (clogDpH6.5, clogP, ASAhydrophobic - accessible molecular surface area of all hydrophobic atoms [29]) and size (Min proj Area - minimum of projection area based on van der Waals radius, and Mvol - molecular volume), but polarizability, ovality and positive charge (ASAplus - water accessible molecular surface area of all atoms with positive partial charge) were also correlated with a high degree of solubilization. Polar surface area, acidity (number of acidic oxygens) and hydrogen bonding capability (δH [25]) were on the other hand correlated to lower SR or solubilization.

4. Conclusions

The presence of endogenous lipidic components *in vivo*, such as bile salts and phospholipids, is beneficial for oral drug absorption, as these components can form mixed micelles able to solubilize lipophilic compounds and subsequently improve oral drug absorption. In the gastrointestinal lumen, variability in the molecular/structural properties or levels of mixed micelles is challenging and may affect not only drug concentrations in the gastrointestinal tract but, also, complicate the outcome of *in vivo* bioavailability/bioequivalence studies. In this study, the impact of increasing levels of bile salts on the 24-hr drug solubility was assessed. Solubility studies revealed pronounced improvement in drug solubility when increasing bile salts concentrations, which greatly depended on drug chemical class and drug physicochemical properties. Hansen solubility parameters can be used to estimate the solubility of a solute in a solvent based on their similarity in terms polarity, dispersion forces and their ability to form hydrogen bonds. The parameters do not take solid state interactions, apparent solubility increase due to ionization or aggregation into account but it may be able to provide additional information on why or why not a drug is strongly solubilized in mixed lipid aggregates present in BDMs.

These solubility data highlight that, *in vivo*, profound changes in drug concentrations would be anticipated due to bile salt variability which need to be further investigated and adequately accounted for when designing *in vitro* or *in vivo* studies. Use of statistical tools (regression plots, multivariate data analysis) revealed the complex nature of drug affinity to mixed aggregates and provided a first insight on the drug cases in which bile salt (level) and/or drug properties (ionization, lipophilicity, molecular weight) are critical and need additional considerations. As enabling strategies are often selected to formulate poorly soluble compounds, future incorporation of formulation (surfactants, lipids) and digestion factors to investigate their interplay on the affinity of lipophilic compounds to mixed lipid aggregates would be an excellent opportunity to expand the current data set to more sophisticated formulations.

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638

Figure captions

Figure 1: Structure of the studied compounds.

Figure 2: Logarithm of solubility ratios of the different bile salt levels on the apparent solubility of neutral drugs (red colour), weak bases (green colour) and weak acids (blue colour). Colour shades represent the biorelevant dissolution media (BDM); gradual changes from light to dark colours indicate media of increasing bile salt concentrations (Mean±SD, n=3).

Figure 3: Drug apparent solubility values of neutral compounds as a function of bile salt concentration. Lines represent the best fit of the regression model. Dashed lines represent the 95% confidence intervals. Boxes indicate the slope of the regression line (Mean±SE, n=3) and the goodness of fit.

Figure 4: Drug apparent solubility values of weak bases as a function of bile salt concentration. Lines represent the best fit of the regression model. Dashed lines represent the 95% confidence intervals. Boxes indicate the slope of the regression line (Mean±SE, n=3) and the goodness of fit.

Figure 5: Drug apparent solubility values of weak acids as a function of bile salt concentration. Lines represent the best fit of the regression model. Dashed lines represent the 95% confidence intervals. Boxes indicate the slope of the regression line (Mean±SE, n=3) and the goodness of fit.

Figure 6: a. Standardized coefficients of the studied variables (and interaction terms) for neutral drugs (red colour), weak bases (green colour) and weak acids (blue colour). * denotes standardized coefficients of 0.8 < VIP < 1 while * denotes standardized coefficients of VIP > 1. (Mean, ± SE). b. Variable importance in the projection (VIP) of the studied variables (and interaction terms) for neutral drugs (red colour), weak bases (green

654 colour) and weak acids (blue colour). Dashed lines represent the criteria for selecting moderately influential (0.8 < VIP < 1) and influential (VIP

655 > 1) variables. (Mean, ± SE).

656

657

Tables

Table 1: Physicochemical properties and solubility values (µM) in $PhB_{pH\ 6.5}$ (Mean±SD, n=3) of the selected compounds for solubility experiments.

Chemical Class	Substance	pKa [a]	% ionized	$clogD_{pH\ 6.5}$ [b]	MW (Da)	T_m (°C) [a]	Solubility in $PhB_{pH6.5}$ (µM)
Neutral	Corticosterone	n.a.	0	2.2	346.5	181	324.4±28.6
	Griseofulvin	n.a.	0	2.5	352.8	220	30.6±3.0
	Danazol	n.a.	0	3.6	337.5	225	1.8±0.0 [31]
	Progesterone	n.a.	0	3.8	314.5	121	28.4±0.3
	Felodipine	n.a.	0	4.8	384.3	145	1.8±0.1
Weak bases	Disopyramide	9.89	99.9%	-0.3	339.5	95	2312.8±159.6
	Dipyridamole	6.20	47.6%	1.7	504.7	163	12.7±0.3
	Haloperidol	8.55	99.1%	2.0	375.9	149	119.6±6.2
	Carvedilol	7.80	98.0%	2.4	406.5	114	113.2±2.5 [31]
	Cinnarizine	7.45	90.0%	4.3	368.6	120	4.1±0.1
	Astemizole	5.34	5.6%	4.4	458.6	173	41.9±0.4 [31]
	Tamoxifen	8.37	98.0%	4.8	371.6	97	1.1±0.3
Weak acids	Indoprofen	4.02	99.7%	0.7	281.3	214	1982.0±287.6
	Naproxen	4.23	99.5%	1.3	230.3	153	3438.8±285.6
	Indomethacin	3.91	99.7%	1.5	357.8	155	987.3±61.6
	Warfarin	4.73	98.3%	2.0	308.4	161	329.8±16.8
	Tolfenamic acid	4.08	99.6%	2.9	261.7	207	126.0±4.4

[a]Experimental values (Ref.[39]), [b]Calculated values (Ref. [39])

662 **Table 2:** Composition of the phosphate buffer ($PhB_{pH6.5}$) and biorelevant dissolution media (BDM). The solubility medium variants (sodium
663 taurocholate and lecithin) are shown in the box.

Component (mM)	**$PhB_{pH\ 6.5}$**	**BDM_L**	**BDM_M**	**BDM_H**
Phosphates	29	29	29	29
Sodium	148	148	148	148
Chloride	106	106	106	106
pH	6.5	6.5	6.5	6.5
Sodium taurocholate	-	0.08	1.50	7.50
Lecithin	-	0.020	0.375	1.875

664

Table 3: Calculated Hansen Solubility Parameters for solvents (media) and solutes (compounds) and ΔHansen values for solvent-solute pairs. The parameters represent intermolecular dispersion forces (δD), polarity forces (δP) and hydrogen bond forces (δH) respectively and ΔHansen similarity between solute and solvent pairs with a low value denoting a similar pair.

Solute/Solvent	**δD ($MPa^{½}$)**	**δP ($MPa^{½}$)**	**δH ($MPa^{½}$)**	**$\Delta Hansen_{water}$**	**$\Delta Hansen_{MLA}$**
Astemizole	20.1	5.9	5.2	39.5	9.5
Carvedilol	20.2	6.5	7.4	37.3	7.8
Cinnarizine	19	1.5	4.2	41.4	12.7
Corticosterone	18.8	8.4	8.4	35.4	4.8
Danazol	18.2	5.7	5.5	38.6	8.6
Dipyridamol	20.5	15.7	10.2	33.6	6.4
Disopyramide	17.9	6.4	4.3	39.4	9
Felodipine	18.5	3.9	6.5	38.2	9.3
Griseofulvin	20	9.8	6.1	37.8	7.1
Haloperidol	19.5	7.2	6.9	37.3	7.1
Indomethacin	20.7	6.5	6.9	38.1	8.7
Indoprofen	20.4	5.5	8.6	36.6	8.2
Naproxen	19.7	4	10.2	35.3	8.3
Progesterone	18.6	5.3	2.7	41.4	11
Tamoxifen	18.4	2.9	3.6	41.3	11.9
Tolfenamic acid	20.4	6.1	9.4	35.7	7.5
Warfarin	22	5	8	38.2	10.8
Water	15.5	16	42.3	0	31.3
TCA	18.6	12.9	12.4	30.7	1.8
Lecithin	16.1	6.4	9.1	34.6	7.1
Lipid aggregate	18.1	11.6	11.7	31.8	0

Figure 1

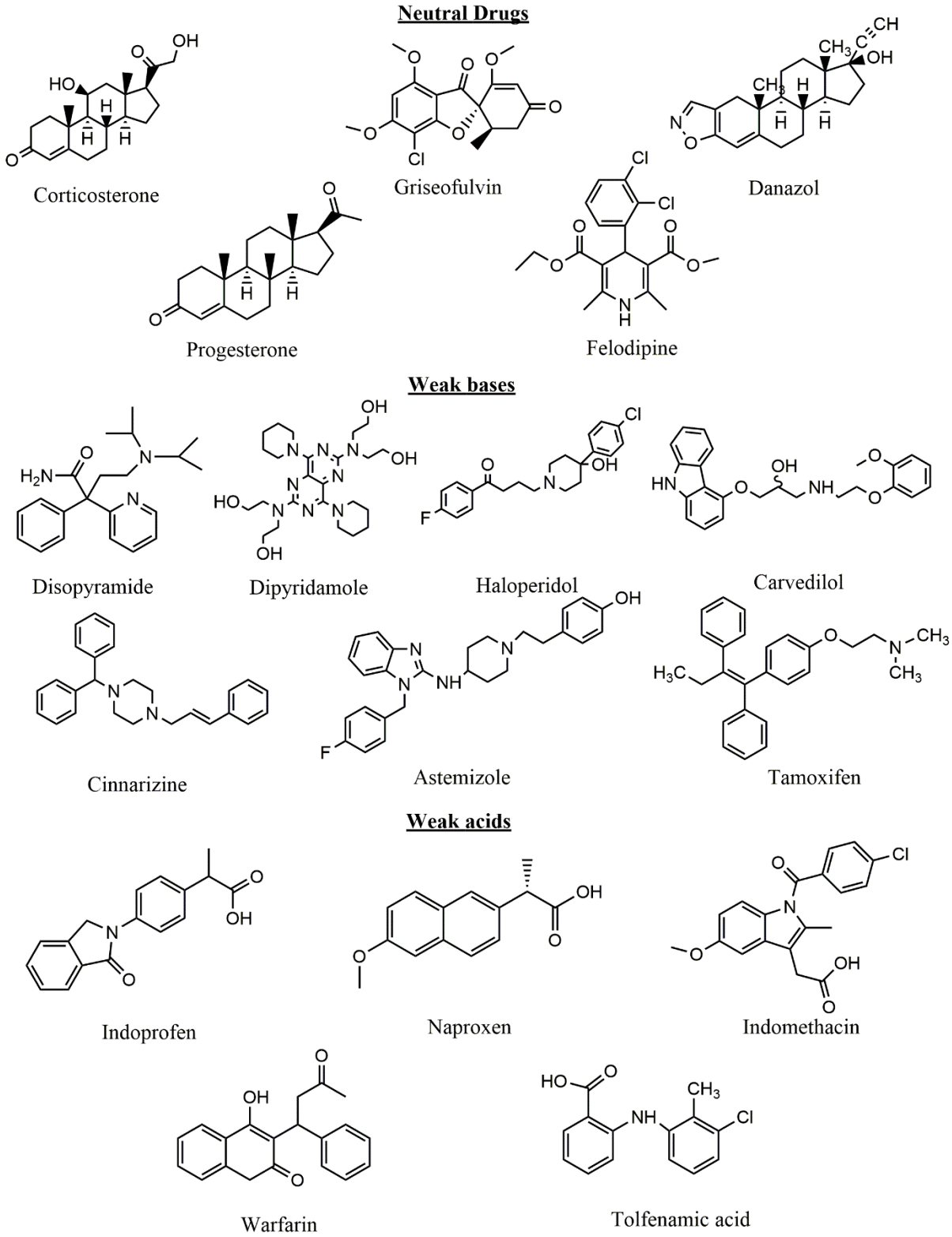

Neutral Drugs
Corticosterone
Griseofulvin
Danazol
Progesterone
Felodipine
Weak bases
Disopyramide
Dipyridamole
Haloperidol
Carvedilol
Cinnarizine
Astemizole
Tamoxifen
Weak acids
Indoprofen
Naproxen
Indomethacin
Warfarin
Tolfenamic acid

Figure 2

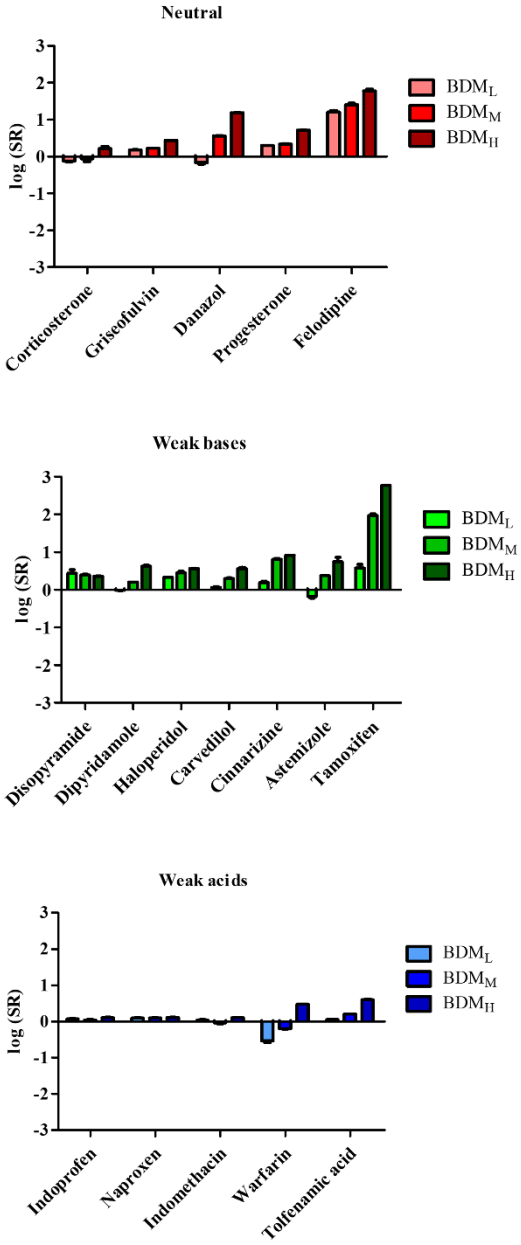

Neutral
log (SR)
3
2
1
0
-1
-2
-3
BDM$_L$
BDM$_M$
BDM$_H$
Corticosterone
Griseofulvin
Danazol
Progesterone
Felodipine
Weak bases
log (SR)
Disopyramide
Dipyridamole
Haloperidol
Carvedilol
Cinnarizine
Astemizole
Tamoxifen
Weak acids
log (SR)
Indoprofen
Naproxen
Indomethacin
Warfarin
Tolfenamic acid

Figure 3

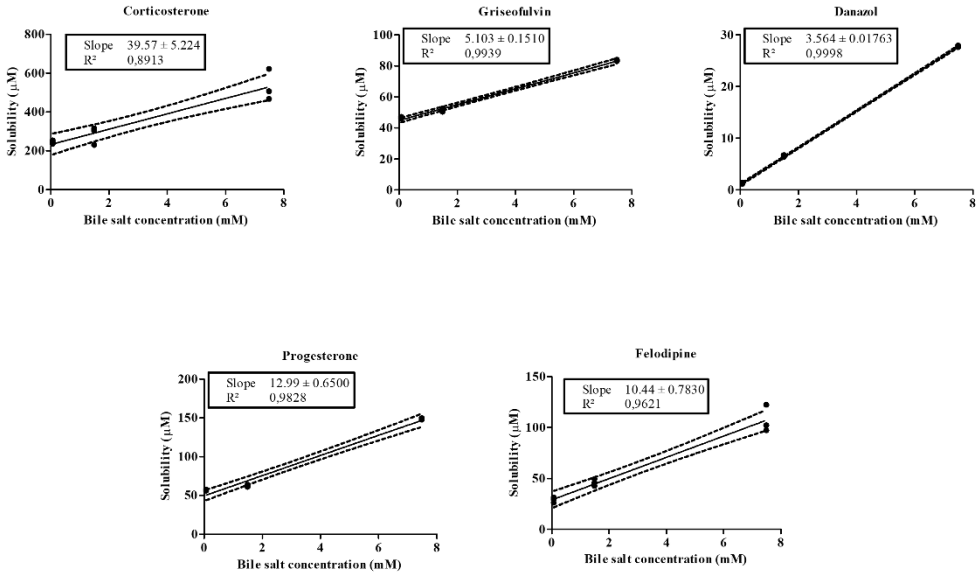

Corticosterone
Slope 39.57 ± 5.224
R² 0,8913
Solubility (µM)
Bile salt concentration (mM)
Griseofulvin
Slope 5.103 ± 0.1510
R² 0,9939
Solubility (µM)
Bile salt concentration (mM)
Danazol
Slope 3.564 ± 0.01763
R² 0,9998
Solubility (µM)
Bile salt concentration (mM)
Progesterone
Slope 12.99 ± 0.6500
R² 0,9828
Solubility (µM)
Bile salt concentration (mM)
Felodipine
Slope 10.44 ± 0.7830
R² 0,9621
Solubility (µM)
Bile salt concentration (mM)

Figure 4

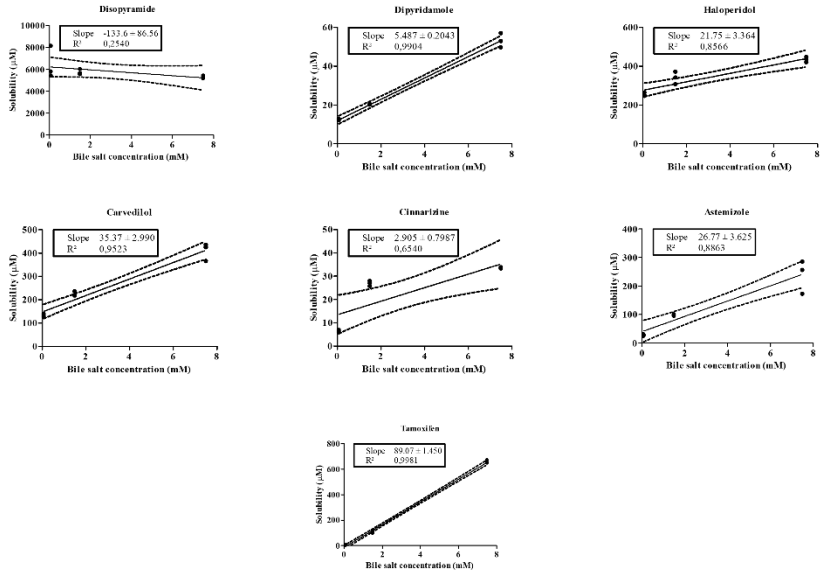

Disopyramide
Slope -133.6 ± 86.56
R² 0.2540
Dipyridamole
Slope 5.487 ± 0.2043
R² 0.9904
Haloperidol
Slope 21.75 ± 3.364
R² 0.8566
Curvedilol
Slope 35.37 ± 2.990
R² 0.9523
Cinnarizine
Slope 2.905 ± 0.7987
R² 0.6540
Astemizole
Slope 26.77 ± 3.625
R² 0.8863
Tamoxifen
Slope 89.07 ± 1.450
R² 0.9981
Solubility (µM)
Bile salt concentration (mM)

Figure 5

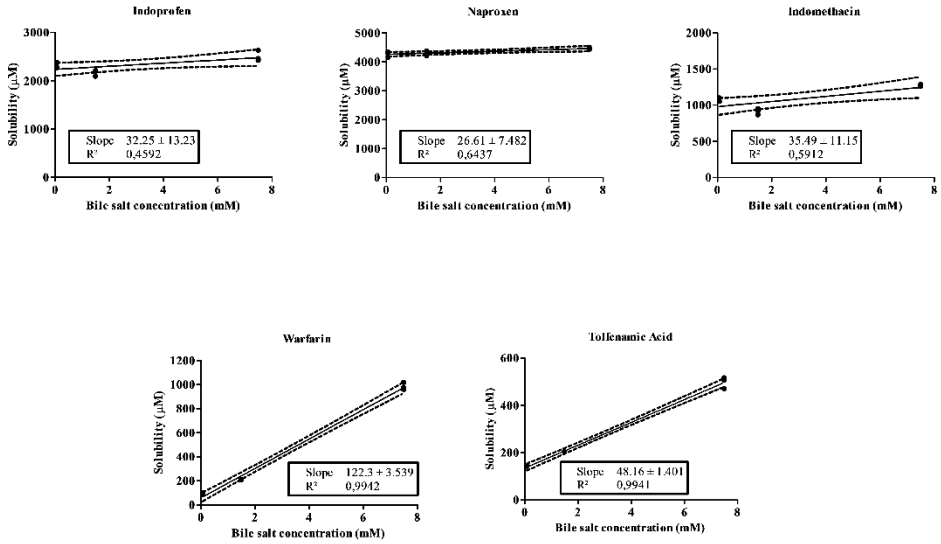
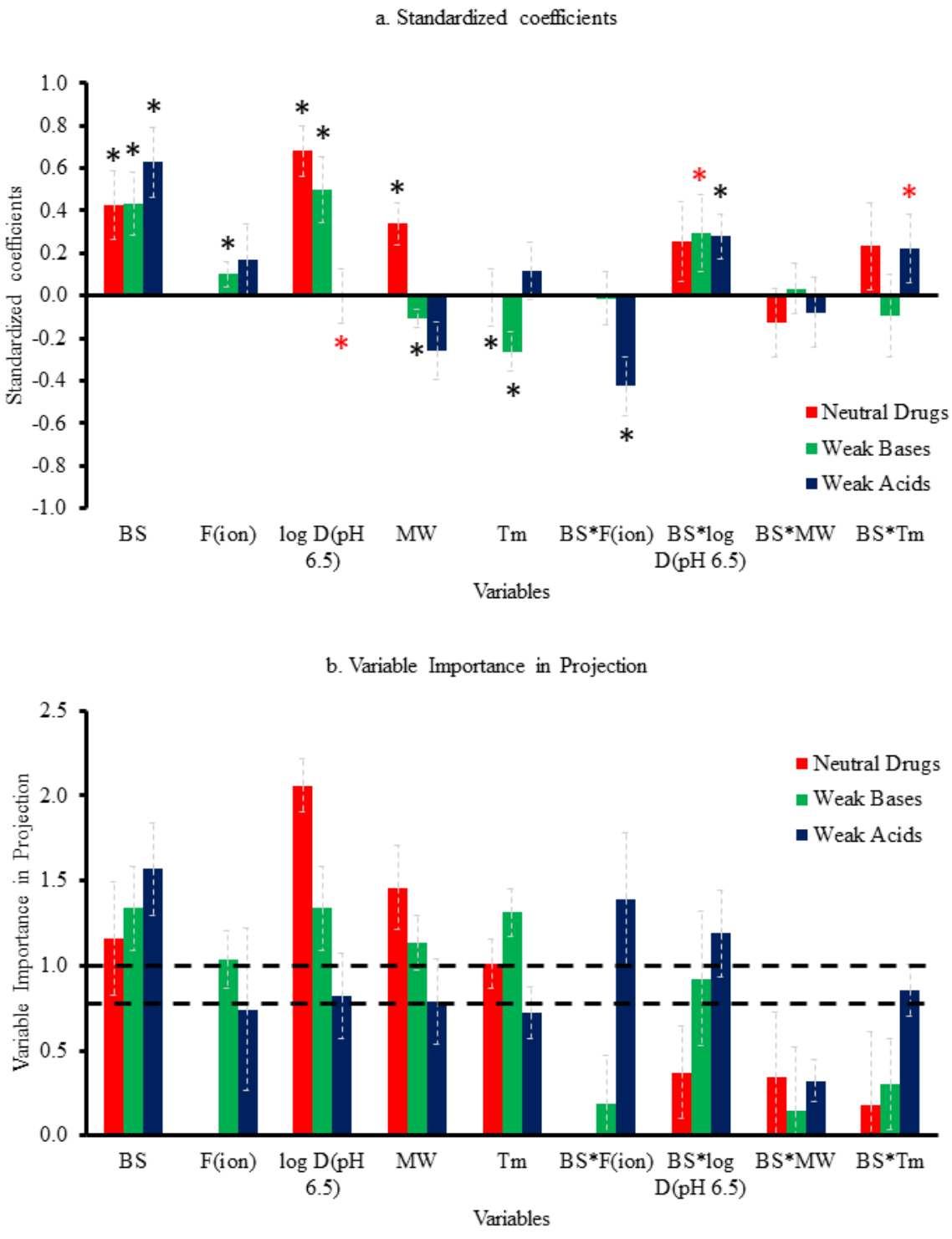

Indoprofen
Solubility (µM)
Slope 32.25 ± 13.23
R² 0,4592
Bile salt concentration (mM)
Naproxen
Solubility (µM)
Slope 26.61 ± 7.482
R² 0,6437
Bile salt concentration (mM)
Indomethacin
Solubility (µM)
Slope 35.49 ± 11.15
R² 0,5912
Bile salt concentration (mM)
Warfarin
Solubility (µM)
Slope 122.3 ± 3.539
R² 0,9942
Bile salt concentration (mM)
Tolfenamic Acid
Solubility (µM)
Slope 48.16 ± 1.401
R² 0,9941
Bile salt concentration (mM)

Figure 6


a. Standardized coefficients
Standardized coefficients
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
BS
F(ion)
log D(pH 6.5)
MW
Tm
BS*F(ion)
BS*log D(pH 6.5)
BS*MW
BS*Tm
Variables
Neutral Drugs
Weak Bases
Weak Acids
b. Variable Importance in Projection
Variable Importance in Projection
2.5
2.0
1.5
1.0
0.5
0.0
BS
F(ion)
log D(pH 6.5)
MW
Tm
BS*F(ion)
BS*log D(pH 6.5)
BS*MW
BS*Tm
Variables
Neutral Drugs
Weak Bases
Weak Acids

Supplementary Material

Supplementary Table 1: Critical molecular descriptors of the studied compounds

Chemical Class	Substance	Polar Surface Area (Å)	Rotatable bonds (counts)	Hydrogen Bond Donors (counts)	Hydrogen Bond Acceptors (counts)
Neutral	Corticosterone	74.60	2	4	2
	Griseofulvin	71.06	3	6	0
	Danazol	46.26	0	3	1
	Progesterone	34.14	1	2	0
	Felodipine	64.63	6	5	1
Weak bases	Disopyramide	59.22	8	4	1
	Dipyridamole	145.44	12	12	4
	Haloperidol	40.54	6	3	1
	Carvedilol	75.74	10	6	3
	Cinnarizine	6.48	6	2	0
	Astemizole	42.32	8	5	1
	Tamoxifen	12.47	8	2	0
Weak acids	Indoprofen	57.61	3	4	1
	Naproxen	46.53	3	3	1
	Indomethacin	68.53	5	5	1
	Warfarin	63.60	4	4	1
	Tolfenamic acid	49.33	3	3	2

Supplementary Table 2: HPLC methods used for drug quantification

Substance	Temp.(º C)	Mobile phase (v/v)	λ (nm)	Range of calibration curves (µM)
Astemizole[1]	40	ACN + formic acid 0.1 % 20:80	283	1.3-43.6
Carvedilol[2]	25	ACN: H_2O + TFA 0.1% 40:60	241	1.5-49.2
Cinnarizine[3]	40	MeOH: H_2O 85:15	252	1.7-27.1
Corticosterone[4]	40	MeOH + formic acid 0.1% 60:40	243	5.4-86.5
Danazol[5]	25	MeOH: H_2O 85:15	285	0.9-29.6
Dipyridamole[2]	25	ACN: H_2O + TFA 0.1% 30:70	280	1.2-39.6
Disopyramide[6]	40	ACN: H_2O + formic acid 0.1% 20:80	261	11.0-176.7
Felodipine [7]	40	MeOH: H2O 75: 25	238	1.6-52.0
Griseofulvin [8]	25	MeOH: H2O 65: 35	292	0.8-14.1
Haloperidol [9]	40	ACN: H_2O + formic acid 0.1% 30:70	230	4.9-79.8
Indomethacin [8]	25	MeOH: H_2O+ formic acid 0.1% 70:30	270	10.4-167.7
Indoprofen [10]	40	ACN: H_2O + formic acid 0.1% 40:60	280	26.6-426.6
Naproxen [11]	40	ACN + 0.1 % formic acid 60:40	239	2.7-86.9
Progesterone [12]	25	MeOH + H2O (70:30)	243	1.0-15.9
Tamoxifen [13]	40	ACN: H_2O + formic acid 0.1% 40:60	237	5.6-80.6
Tolfenamic acid [14]	40	MeOH: H_2O +formic acid 0.1% 80:20	286	4.7-76.4
Warfarin [15]	25	ACN: H_2O + 0.1% TFA 50:50	280	12.2-194.5

The column used for all tested drugs was, Waters Xbridge Shield RP18, 130Å, 150 X 4.6, 3.5 µm. The injection volume used was 100 µL for all drugs except for Indoprofen, 50 µL. Used flow rate was 0.8 mL/min for Griseofulvin and 1 mL/min for all other drugs.

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